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Studies on the Role of Host Resistance in the Therapy of Mouse Ascites Hepatoma MH 134

by

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I. INTRODUCTION

One outstanding characteristic of malignant neoplasma has generally been accepted to be their ability to grow beyond the control of the host. In recent years, the tumor-specific antigens have been clearly demonstrated in virus-induced and in chemically or physically induced tumors[99)100)]. Some of the cases of spontaneous regression of primary or metastatic tumors would point to the presence of immunologic phenomena[20)28)29)39)105)]. These antigenic tumors can be inhibited better by lymphoid cells[66)] than by serum, with the exception of leukemia E. L. 4[41)]. These observations pose a problem as to why antigenic tumors establish themselves and grow beyond the control of the host.

Therefore, it is very interesting to consider the depressed immunological response and the reinforcement of the immune response in the host. The author investigated the depression of the immunological reaction caused by antitumor drugs and by the tumor itself, then attempted to reinforce the immune response by inoculation with BCG and with spleen cells.

It was found that tumor growth could be delayed by injection of sensitized spleen cells. The probable mechanism of action is discussed.

II. EFFECT OF ANTITUMOR DRUGS ON IMMUNE RESPONSES

A. Titration of hemolysin

Materials and methods

Sheep red blood cells were harvested in Alsver's solution, and 0.5 ml of a 4 % suspension of washed sheep cells in saline was injected intraperitoneally in pure strain C3H/HeMs (♀) mice, 6 to 8 weeks old, supplied by the Animal Center of Kyoto University. Serum was obtained every four days from the hearts of six mice which served as controls (a). From six mice treated (b) with mitomycin C (Kyowa Hakko Kogyo Co. Ltd.) or nitromin (Yoshitomi Pharm. Ind., Ltd.), serum was obtained 11 days after antigen injection. The cytocidal effects of these drugs on MH 134 cells have been confirmed[27)112)]. Hemolysin titers were determined by the method of MALMGREN et al.[79)] at half of its volume. Mitomycin C was injected intraperitoneally in dose of 10 γ a day for 3 days before the antigen, and 10 γ a day for 3 or 5 days after the antigen injection. Nitromin was injected in dose of 200 γ a day intraperitoneally for 3 days before and 3 days after the antigen injection.

Results

(a) Control

High hemolysin titers were observed 9 and 12 days after antigen injection.

The results are shown in Table 1. and Fig. 1.

(b) Treatment with mitomycin C or nitromin

Treatment with both nitromin and mitomycin C resulted in a decrease in hemolysin titers as shown in Table 2 and Fig. 1.

Mitomycin C suppressed hemolysin titer more before than after antigen injection in equal dose, while nitromin suppressed it more after than before antigen injection.

B. Delayed type hypersensitivity

(a) preliminary experiment

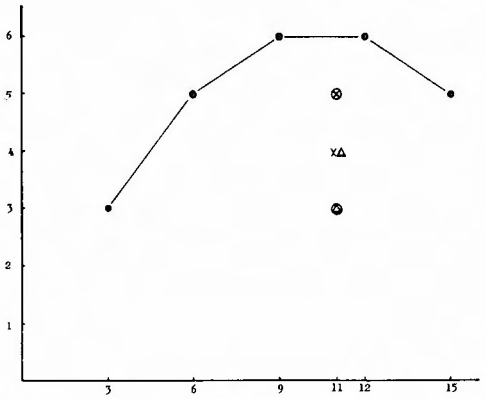

Fig. 1 Hemolysin titers.
$D = 5 \times 2^{y-1}$, herein D shows maximum serum dilution for complete hemolysis. —•—•— : Control × : Nitromin. △ : Mitomycin C. ○ : Before antigen injection.

Table 1 Hemolysin titers in non-treated C3H mice

Days after antigen injection	Hemolysis* by dilution of antisera									
	1 : 5	1 : 10	1 : 20	1 : 40	1 : 80	1 : 160	1 : 320	C_1	C_2	C_3
3	0	0	0	1	2	3	4	4	4	4
6	0	0	0	0	0	1	2	4	4	4
9	0	0	0	0	0	0	1	4	4	4
12	0	0	0	0	0	0	1	4	4	4
15	0	0	0	0	0	1	2	4	4	4

* : Zero indicates complete hemolysis and 4 indicates no hemolysis ; 1, 2, 3 representing intermediate grades of hemolysis.

C_1 : Control without complement. C_2 : Control without hemolysin.

C_3 : Control without complement and hemolysin.

Table 2 Effect of mitomycin C or nitromin on hemolysin titers

Drug	Dose	Hemolysis* by dilution of antisera									
		1 : 5	1 : 10	1 : 20	1 : 40	1 : 80	1 : 160	1 : 320	C_1	C_2	C_3
Mitomycin C	30 γ before antigen	0	0	0	1	3	4	4	4	4	4
	30 γ after antigen	0	0	0	0	1	3	4	4	4	4
	50 γ after antigen	0	0	2	4	4	4	4	4	4	4
Nitromin	600 γ before antigen	0	0	0	0	0	1	2	4	4	4
	600 γ after antigen	0	0	0	0	1	2	3	4	4	4

* : Zero indicates complete homolysis and 4 indicates no hemolysis ; 1, 2, 3 representing intermediate grades of hemolysis.

C_1 : Control without complement. C_2 : Control without hemolysin.

C_3 : Control without complement and hemolysin.

Materials and methods

According to the slightly modified method of KOLIN et al.,[69] white guinea pigs weighing about 300 g were sensitized by injecting into each paw 0.1 ml of an emulsion of Freund's incomplete adjuvant or complete adjuvant (Iatron Laboratories, Tokyo) with an equal volume of ovalbumin. The total sensitizing dose was 5 γ of ovalbumin. On the 6th day after sensitization, the guinea pigs were tested by the intradermal injection of 50 γ of ovalbumin in 0.1 ml of saline. Tests were read at 6 and 24 hours as advocated by FRIEDMAN et al.[34] and the results were recorded in terms of the diameters of erythema.

Results

None of the animals showed any erythema at 6 hours. The size of the skin reaction at 24 hours is noted in Table 3.

Table 3 Size (mm) of erythema at 24 hours following skin test

	Size (mm) of erythema			
Incomplete adjuvant	B_1*	B_2	B_3	B_4
	2×2	5×6	4×5	6×6
Complete adjuvant	B_5	B_6	B_7	B_8
	11×13	14×17	14×16	11×15

* : Guinea pig

When complete adjuvant was used the area of erythema was large and definite.

(b) Effect of antitumor drugs

Materials and methods

White guinea pigs, weighing 300 to 400 g., were divided into 3 groups. Two groups of animals were injected intraperitoneally every day with 50 γ of mitomycin C or 1.25 mg of nitromin. The control group was injected with saline. Sensitization with ovalbumin and Freund's complete adjuvant and the observation of erythema at 24 hrs. were carried out as described in the preliminary experiment. Treatment was started 2 days before the inoculation of antigen and continued for 8 days.

Results

The diameters of erythema are shown in the Table 4 and their mean values are compared.

Generally sample means are represented by x and y and the difference between means can be tested according to the value of F_s calculated from the following formula, granted a homogeneous variance in the distribution of F[124].

$$F_s = \frac{(\bar{x}-\bar{y})^2}{\omega^2\left(\frac{1}{N_1}+\frac{1}{N_2}\right)} \qquad \omega_2 = \frac{S_x+S_y}{N_1+N_2-2}$$

Table 4 Size (mm in diameter) of erythema in different treatments and summary of statistics for comparison

Treatment	Size (mm)	Mean (mm)	Sum of squares	Degrees of freedom	Mean square
Nitromin	22 0 19 4 14	11.8	360.8	4	90.2
Mitomycin C	17 5 30 18	17.5	313.0	3	104.3
Saline	25 33 38 35 20 28	29.8	226.9	5	45.3

Here S_x and S_y denote the sums of squares and N_1 and N_2 the size of the group x and y. Comparing the effect of treatment with the control, the variance ratio $=1.9<F_5^4$ (0.025) $=7.39$ with nitromin and $2.3<F_5^3$ (0.025) $=7.76$ with mitomycin. The variance was thus noted to be homogeneous[81)115)], and F_s was calculated. Since it was $13.5>F_{6+5-2}^1$ (0.01) $=10.56$ with nitromin and $5.38>F_{6+4-2}^1$ (0.05) $=5.32$ with mitomycin, the differences between mean diameters were significant. The significant suppression of the skin reaction by the antitumor drugs was thus indicated. Subsequently, the difference between nitromin and mitomycin was tested. The variance ratio was $1.1<F_4^3$ (0.025) and $F_s=0.75<F_{5+4-2}^1$ (0.05). The difference was not significant.

III. DEPRESSED IMMUNOLOGICAL REACTIVITY IN TUMOR-BEARING HOSTS

Materials and methods

Male C3H mice ($H\text{-}2^k$), about 8 weeks old, were divided into three groups. The suspensions containing 10^6 MH 134 cells were injected intraperitoneally into those with the ascitic form and subcutaneously into those with the subcutaneous form, Skin form male BALB/C mice ($H\text{-}2^d$) was transplanted 2 days and 5 days later, respectively. One group served as the control. The transplantation was performed according to the method of BILLINGHAM and his colleagues[13)14)]. The abdominal skin of BALB/C mice was outlined with a cork borer (1 cm in diameter), and full-thickness skin grafts were prepared. The grafts were transplanted to the raw area on the chest of C3H mice. Dressings were removed 6 days after grafting. The survival time of the graft was determined as the beginning of the necrosis by naked eye inspection.

Results

The logarithmic transformation is generally used for the distribution of survival time[6)]. The author compared the mean survival time in days with that of the control in logarithms (log.).

Table 5 shows the survival time and statistical analysis. The variance ratios represent the value of the larger mean square over the smaller between the treated group and the controls. The test of homogeneity of variance showed $1.2<F_5^{11}$ (0.025) and $1.3<F_{11}^7$ (0.025), therefore, they were homogeneous. Then, the differences in mean survival time between the ascitic or subcutaneous form and the controls were tested according to the formula previously described. F_s was 21.1 in the former and 24.7 in the latter. $21.1>F_{12+6-2}^1$ (0.01) $=8.53$ $24.7>F_{12+8-2}^1$ (0.01) $=8.28$. Therefore, these differences were

Table 5 Survival time of skin homografts in C_3H mice and summary of statistics for comparison

Group	Survival time (days)	Mean log.	Sum of squares	Degree of freedom	Variance ratio
Ascitic form	11 11 12 13 13 15	1.09	0.01393	5	1.2
Subcutaneous form	11 11 11 12 12 13 14 17	1.10	0.03099	7	1.3
Control	7 8 8 9 9 9 9 10 10 10 11 11	0.96	0.03729	11	

significant. The significant prolongation of skin graft survival was indicated.

IV. REINFORCEMENT OF IMMUNOLOGICAL CAPACITY IN TUMOR-BEARING HOSTS

A. Transplantation of ascites hepatoma MH 134 after BCG inoculation

Materials and methods

Male mice of the C3H strain, 6 to 8 weeks old, were divided into 2 groups of 4 mice each. One group was injected intraperitoneally with 0.05 mg of BCG (Japanese BCG Co.), and the other group served as controls. Eight days later, 10^6 tumor cells were injected subcutaneously. Their growth was checked and recorded by measuring the diameter of each tumor by calipers.

Results

The tumor diameters (mm) and survival time are shown in Table 6.

The growth rates of the developing tumors are presented in Fig. 2, which shows that tumors in mice treated with BCG grew more slowly than in the controls. Next a test for the difference of the mean vector was carried out in the distribution of F., with the formula

$$F_s = \frac{(M+N-k-1)}{k(M+N)} \sum_{\alpha=1}^{k} \sum_{\beta=1}^{k} \varphi_{\alpha\beta} (\bar{x}_{\cdot\alpha} - \bar{y}_{\cdot\alpha})(\bar{x}_{\cdot\beta} - \bar{y}_{\cdot\beta})$$

$$\alpha = 1, 2, \cdots\cdots \alpha, \beta \cdots\cdots k \qquad \beta = 1, 2, \cdots\cdots \alpha, \beta \cdots\cdots k$$

where $\bar{x}_\alpha = \frac{1}{N} \sum_{i=1}^{N} x_{i\alpha}$, and $\bar{y}_\beta = \frac{1}{M} \sum_{i=1}^{M} x_{i\beta}$

This value[64)24)] has the distribution of F with $n_1 = k$ and $n_2 = M+N-k-1$ degrees of freedom. The letter $\varphi_{\alpha\beta}$ is defined as follows:

$$\sum_{\beta=1}^{k} \phi_{\alpha\beta} \varphi_{\beta\gamma} = \Sigma_{\alpha\beta} \qquad \begin{cases} \text{if } \alpha = \gamma, & \delta = 1 \\ \text{if } \alpha = \gamma, & \delta = 0 \end{cases}$$

$$\alpha, \gamma = 1, 2 \cdots\cdots k$$

$$\phi_{\alpha\beta} = \sum_{i=1}^{M} (x_{i\alpha} - \bar{x}_{\cdot\alpha})(x_{\cdot\beta} - \bar{x}_{\cdot\beta}) + \sum_{i=1}^{M} (y_i\alpha - \bar{y}_0\alpha)(y\beta - \bar{y}_0\beta)$$

Table 6 Tumor size (mm in diameter) after inoculation

Group	Mice	Tumor size (mm) at days#				survival time (days)
		7	14	20	27	
Treatment with BCG	B_1	6	13	15		23
	B_2	5	11	14	21	34
	B_3	5	10	12	16	36
	B_4	6	10	15	21	30
Control*	B_5	7	12	16	21	29
	B_6	8	13	16	23	27
	B_7	5	11	15	18	30
	B_8	9	13	16		

* : Non-treated mice

\# : After BCG inoculation

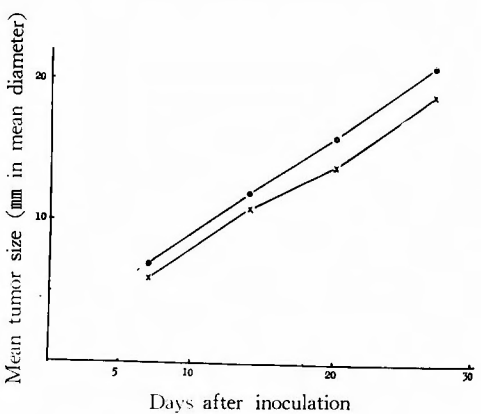

Fig. 2 Growth rate of tumors.
—•—•— : Control. —×—×— : Group injected with BCG.

For the calculation k was selected on the 7th, 14th and 20th day, then k=3.

$\varphi_{11}=0.181$ $\varphi_{12}=-0.0911$ $\phi_{13}=-0.0559$ $\varphi_{22}=0.210$ $\varphi_{23}=-0.0738$ $\varphi_{33}=0.230$

$$F_s=\frac{(4+4-3-1)}{3(4+4)} \quad 4\times4\times0{,}523=1.39$$

$$F^3_{4+4-3-1}\ (0.05)=6.59$$

The difference was not signifficant. Next the test of the difference between mean survival times was carried out by the usual logarithmic transformation.

In the computation, the sum of squares was 0.02490 in the BCG treated group and 0.01148 in the controls. Then, the ratio of variance=$2.1<F^3_5\ (0.025)=15.44$. The variances were thus homogeneous. Then, the value of $F_s=1.11<F^1_{4+4-2}\ (0.05)=5.99$. $Pr\{F>F_s\}>5\%$ The difference between mean survival times was not significant.

B. Transfer of immunologically competent cells to the tumor-bearing host

Materials and methods

As a source of immunologically competent cells, normal or tumor-sensitized spleens of male C3H mice, about 8 weeks old, were used. Sensitization was performed 4 weeks before extirpation of spleen by the inoculation of 2×10^6 MH 134 cells killed in vitro by incubation with 500 γ of mitomycin C per 10^6 tumor cells for 100 min. at 37°C. The spleens were minced in Hanks' solution and the cells were harvested from the supernatant of the suspension. Then 2×10^6 normal or sensitized spleen cells were injected intraperitoneally in male mice 6 or 4 days after the intraperitoneal inoculation of 10^6 MH 134 cells.

Results

The results of the experiment are summarized in Table 7, and a statistical analysis was carried out.

Table 7 Effect of transfer of spleen cells on survival time

Mice	Treatment									Control* Survival time (days)				
	Number of Spleen cells	Type of spleen cells	Days after inoculation	Survival time (days)										
5-week-old	2×10^6	normal	6	13	14	15	15	16		13	13	13	14	14
8-week-old	2×10^6	normal	6	18	18	18	18	19		16	16	17	17	18
5-week-old	2×10^6	tumor-sensitized	4	10	14	16	16	16		10	11	13	14	16

* : Non-treated tumor-bearing mice.

The survival times were transformed to logarithms as usual. In the group of mice, 5 weeks old, injected with normal spleen cells, the sum of squares (S) was 0.00492 in the treated group and 0.00192 in the controls. Then, the homogeneity of variance was tested. The ratio of variance=$2.5<F^4_4\ (0.025)=9.60$. The homogeneity was verified. The difference of mean values was tested in F-distribution.

$$\omega^2=0.000855 \qquad F_s=4.22<F^1_{5+5-2}\ (0.05)=5.32$$

The difference in the mean survival time between animals treated with normal spleen cells

and untreated controls was not significant. On the other hand, in 8-week-old mice, the data lead to the following analysis. S. was 0.00032 in the treated group and 0.00252 in the control. From Ratio of Variance $=9.6<F_4^4$ (0.025), the homogeneity was verified.

$$F_s=11.2>F_{5+5-2}^1 \ (0.05). \quad Pr\{F>F_s\}< 5\ \%.$$

There was thus a significant difference in the mean survival time of 8-week-old mice. The significant prolongation of survival time was thus indicated. In mice injected with tumor-sensitized spleen cells, S. was 0.03968 in the treated group and 0.02648 in the controls. The ratio of variance was $1.4<F_4^4$ (0.025). In the test of difference in mean survival time in F-distribution, $F_s=0.204<F_{5+5-2}^1$ (0.05). There was no difference in the survival times between animals treated with tumor-sensitized spleen cells and the controls.

C. Transplantation of MH 134 cells after exposure to spleen cells

(a) Preliminary experiment: Survival rates of cells

Materials and methods

By the method of SCHRECK[113], tumor cells and spleen cells were stained with eosin yellow in Hanks' solution containing penicillin 200U/ml and streptomycin 200 γ/ml.

Results

Table 8 shows the survival rates of these cells. Each experiment was designed to finish within 3 hours.

Table 8 Survival rates of tumor cells and spleen cells

Time	Survival rates (%) at room temperature	
	MH 134	Normal spleen cells
2 hours	94	84
3 hours	88	69
4 hours	84	52

(b) Inoculation of MH 134 cells exposed to tumor-sensitized allogeneic spleen cells

Materials and methods

Female BALB/C mice were injected intraperitoneally with about 2×10^6 tumor cells. Twelve days later, the spleen cells were harvested as usual. The mixture of 10^6 tumor cells and 3×10^6 spleen cells per 0.2 ml in Hanks' solution, and the tumor suspension of 10^6 cells per 0.2 ml in Hanks' solution were prepared. They were incubated at 37°C for 60 min. Subsequently 0.2 ml of each suspension was injected subcutaneously in the right inguinal area or intraperitoneally to 6 to 8-week-old male C3H mice. Developing tumors were measured by calipers and the survival time was recorded.

Results

The tumor diameters (mm) are summarized in Table 9. The growth curves of the tumor are illustrated in Fig. 3. Treatment with tumor-sensitized spleen cells resulted in prolonged latency periods and reduced growth rates. The graphical estimation was analysed in the distribution of F.

$$F_s=\frac{(M+N-k-1)}{k(M+N)}M\cdot N\sum_{\alpha=1}^{k}\sum_{\beta=1}^{k}\varphi_{\alpha\beta}(\bar{x}_{\cdot\alpha}-\bar{y}_{\alpha})(\bar{x}_{\cdot\beta}-\bar{y}_{\cdot\beta})$$

where k was selected on 10th, 17th and 28th day. Consequently k was 3, and $M=N=4$.

$$F_s=21.3>F_{4+4-3-1}^3 \ (0.01)=16.69$$

The difference of mean vectors in development was significant. The survival time for the ascitic form is shown in Table 10.

Table 9 Tumor size (mm) after inoculation

Group	Mice	Tumor size (mm) at days#								Survival time (days)
		10	13	17	21	23	28	31	33 (days)	
Treatment with spleen cells	B_1	0	3	7	8	10	15	18	20	33
	B_2	0	2	4	7	10	20	20	22	33
	B_3	0	0	3	6	7	11	12	17	33
	B_4	0	2	4	7	9	17	17	20	33
Control*	B_5	4	6	13	15	20	20	21		31
	B_6	5	10	14	18	20	24			28
	B_7	3	4	10	12	17	19	20		33
	B_8	5	8	13	16	19	22			30

* : Mice inoculated with incubated tumor suspension alone.
\# : After inoculation.

The ratio of variance was $2.2 < F_5^5\ (0.025) = 7.15$. Then F_s in difference was calculated.

$$F_s = 76.1 > F_{10}^1\ (0.01) = 10.04$$

The difference between mean survival time for the ascitic form was significant. The significant suppression of tumor growth and prolongation of survival time were thus indicated.

(c) Inoculation of MH 134 cells exposed to isogeneic BCG-sensitized spleen cells

Materials and methods

Male C3H mice were injected intraperitoneally with 0.05 mg of dry BCG, and 20 days later, the spleen cells were harvested aseptically in Hanks' solution. A mixture of 10^6 tumor cells and 6×10^6 spleen cells per 0.2 ml in Hanks' solution, and a tumor suspension of 10^6 cells per 0.2 ml in the same solution were prepared. They were incubated at 37°C, for 50 min. Subsequently, 0.2 ml of each suspension was injected intraperitoneally in 6 to 8-week-old male C3H mice. The survival times were observed.

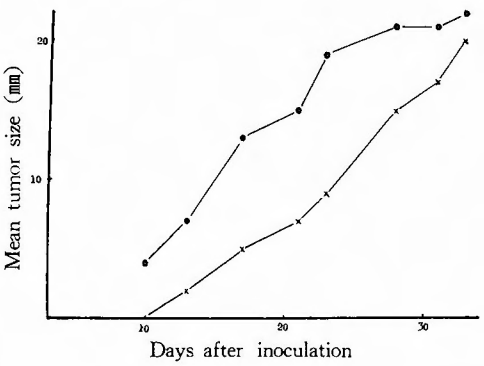

Fig. 3 Growth rate of tumors.
—·—·— : Control. —×—×— : Group treated with tumor-sensitized allogeneic spleen cells.

Table 10 Effect of tumor-sensitized spleen cells on survival time for ascitic form

Group	Survival time (days)	Mean log.	Sum of squares	Degrees of freedom	Variance ratio
Treatment with spleen* cells	18 19 20 20 21 23	1.33	0.00593	5	2.2
Control#	11 12 12 13 14 15	1.11	0.01314	5	

* : Spleen cells from BALB/C mice.
\# : Mice inoculated with incubated tumor suspension alone.

Results

Table 11 shows the survival time in days.

Table 11 Effect of BCG-sensitized spleen cells on survival time for ascitic form

Group	Survival time	Mean log.	Sum of squares	Degrees of freedom	Variance ratio
Treatment with spleen cells*	19 19 20 21 26 31	1.35	0.03633	5	18
Control#	14 14 15 15 15 16	1.17	0.00193	5	

* : Spleen cells from C_3H mice.

\# : Mice inoculated with tumor suspension alone.

Variance ratio $=18>F_5^5\ (0.025)=7.15$.

The variance was not homogeneous. The groups were of the same size, the formula was calculated and tested in the F-table with degrees of freedom $=N_1-1$ instead of N_1+N_2-2.

$$Fs=25.3>F_5^1\ (0.01)=16.26$$

The difference between mean survival times was significant. The significant prolongation of survival time was indicated.

V. COMBINED TREATMENT WITH IMMUNOLOGICALLY COMPETENT CELLS AND ANTITUMOR DRUGS

Materials and methods

To avoid immunological depression by the drug, the author transplanted MH 134 cells exposed to immunologically competent cells, later treated with nitromin. (A) The use of tumor-sensitized allogeneic spleen cells. About 2×10^6 MH 134 cells were injected once intraperitoneally in female BALB/C mice, and 14 days later, sensitized cells were harvested from the minced spleens in medium 199 containing antibiotics and 20% calf serum. (B) The use of BCG-sensitized isogeneic spleen cells. Male C3H mice were injected intraperitoneally with 0.1 mg of dry BCG and 18 days later spleen cells were harvested in the same manner.

Mixtures of 10^6 tumor cells and 2×10^6 spleen cells per 0.2 ml in medium and the tumor suspensions of 10^6 cells per 0.2 ml were incubated at 37°C for 50 min. Then 0.2 ml of each suspension was injected subcutaneously or intraperitoneally in 6 to 8-week-old C3H mice of the same sex. Subsequently 20 γ of nitromin was injected intraperitoneally to them on the 8th and 10th days. The controls were treated with nitromin alone. Developing tumors were measured by calipers and the survival times were noted.

Results

A. The use of tumor-sensitized allogeneic spleen cells

(a) Subcutaneous form

The tumor size (mm in diameter) are shown in Table 12.

The growth curves of the tumors are presented in Fig. 4, showing that tumors treated with spleen cells grew more slowly than the controls. Table 13 (a) summarizes the survival time and statistical analysis of the subcutaneous form.

Table 12 Tumor size (mm) after inoculation in combined treatment

Group	Mice	Tumor size (mm) at days#							Survival time (days)
		7	11	16	20	23	26	30 (days)	
Treatment with spleen cells §	B_1	5	6	10	13	15	16	18	36
	B_2	5	7	11	13	16	19		28
	B_3	2	4	7	10	13	15	16	41
	B_4	4	6	10	12	15	17	20	31
	B_5	0	3	8	11	13	16	18	41
Control*	B_6	7	9	11	13	16			23
	B_7	5	7	12	15	18			25
	B_8	8	12	13	15	17	20		27
	B_9	8	11	13	17	20			25
	B_{10}	7	10	12	16	18	21		26

§ : Tumor-sensitized spleen cells from BALB/C mice.
* : Mice treated with nitromin alone.
# : After inoculation.

In the subcutaneous form the variance ratio $=6.0<F_4^4\ (0.025)=9.60$, and the variance was homogeneous.

The difference between mean survival time was calculated according to the usual formula, where $\omega^2=0.00230$, $F_s\ 24.4>F_{5+5-2}^1\ (0.01)=11.26$

The difference was significant.

(b) Ascitic form

Table 13 (b) summarizes the survival time and statistical analysis.

In the ascitic form, the variance ratio $=4.1<F_6^6\ (0.025)=5.82$.

The variance was homogeneous

$\omega^2=0.00302$ $Fs=16.6>F_{7+7-2}^1\ (0.01)=9.33$.

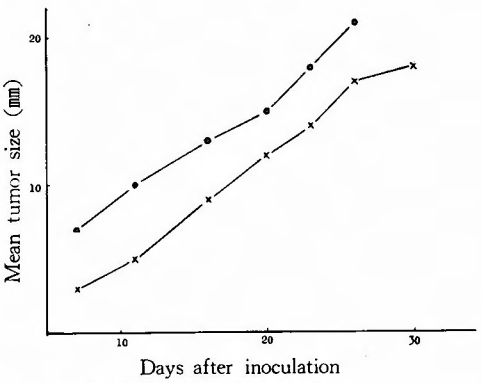

Fig. 4 Growth rate of tumors.
—•—•— : Control. treated with nitromin.
—×—×— : Group treated with tumor-sensitized allogeneic spleen cells and nitromin.

The difference between mean survival times was significant. The suppression of tumor growth on the graph and the significant prolongation of survival time in both forms were indicated.

Table 13 (a) Effect of tumor-sensitized spleen cells for subcutaneous form in combined treatment

Group	Survival time (days)	Mean log.	Sum of squares	Degrees of freedom	Variance ratio
Treatment with spleen cells*	36 28 41 31 41	1.55	0.01580	4	6.0
Control	23 25 27 25 26	1.40	0.00260	4	

* : Spleen cells from BALB/C mice.

Table 13 (b) Effect of tumor-sensitized spleen cells for ascitic form in combined treatment

Group	Survival time (days)							Mean log.	Sum of squares	Degrees of freedom	Variance ratio
Treatment with spleen cells#	20	20	20	21	23	23	25	1.33	0.00709	6	4.1
Control*	13	14	16	16	18	19	20	1.21	0.02918	6	

* : Mice treated with nitromin alone.
\# : Spleen cells from BALB/C mice.

B. The use of BCG-sensitized isogeneic spleen cells

(a) Subcutaneous form

Table 14 shows the size of the developing tumors

Table 14 Tumor size after inoculation in combined treatment

Group	Mice	Tumor size (mm) at days#							Survival time (days)
		5	7	10	13	16	20	24 (days)	
Treatment with spleen cells §	B_1	0	3	7	11	12	14	17	28
	B_2	3	5	8	13	15	19		23
	B_3	5	7	10	15	18			18
	B_4	2	5	9	12	15	17	20	25
	B_5	3	5	7	10	12	15	18	28
Control*	B_6	3	6	8	11	14	19		20
	B_7	5	8	11	15	18			18
	B_8	5	7	10	13	15	18		22
	B_9	3	5	8	11	12	16	20	27
	B_{10}	4	6	9	12	14	18	21	24

§ : BCG-sensitized spleen cells from C_3H mice.
* : Mice treated with nitromin alone.
\# : After inoculation.

The growth curves of the tumors are presented in Fig. 5, which shows that the treated group grew more slowly than the controls. The difference between survival times was analysed as in Table 15 (a).

In the subcutaneous form, the variance ratio $1,4 < F_4^4\ (0.025) = 9.60$.

The variance was homogeneous.

$$F_s = 0.75 < F_{5+5-2}^1\ (0.05) = 5.32.$$

The difference was not significant.

(b) Ascitic form

Table 15 (b) shows their survival times. In the ascitic form, the variance ratio $= 1.0 < F_5^5\ (0.025) = 7.15$ The variance was homogeneous.

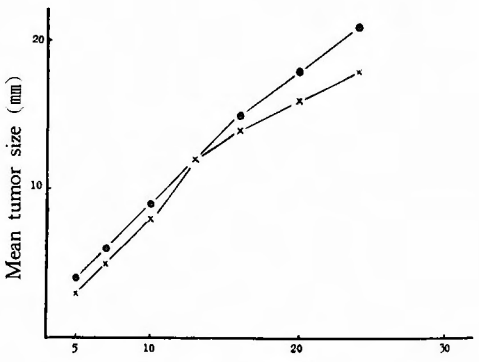

Fig. 5 Growth rate of tumors.
—•—•— : Control treated with nitromin.
—×—×— : Group treated with BCG-sensitized isogeneic spleen cells and nitromin.

Table 15 (a) Effect of BCG-sensitized spleen cells for subcutaneous form in combined treatment

Group	Survival time (days)	Mean log.	Sum of squares	Degrees of freedom	Variance ratio
Treatment with spleen cells*	28 23 18 25 28	1.38	0.02492	4	1.4
Control	20 18 22 27 24	1.34	0.01765	4	

* Spleen cells from C_3H mice.

Table 15 (b) Effect of BCG-sensitized spleen cells for ascitic form in combined treatment

Group	Survival time (days)	Mean log.	Sum of squares	Degrees of freedom	Variance ratio
Treatment with spleen cells#	15 15 18 20 21 22	1.26	0.02434	5	1.0
Control*	12 13 14 15 17 18	1.17	0.02389	5	

* : Mice treated with nitromin alone.

\# : Spleen cells from C_3H mice.

$$F_s = 5.04 > F^1_{5+5-2}\ (0.05) = 4.96$$

The difference was significant.

The only slight suppression of tumor growth on the graph and the significant prolongation of survival time in the ascitic form were indicated.

VI. OBSERVATION IN TISSUE CULTURE

Materials and methods

Female BALB/C mice (A) were injected intraperitoneally with 2×10^6 MH 134 cells 1 time or 4 times, and the male C3H mice (B) were sensitized with 0.1 mg of BCG. Spleen cells were harvested 14 days after the last sensitization with MH 134 cells and 18 days after sensitization with BCG. Medium 199 containing penicillin (200 u/ml) and streptomycin (200 γ/ml) was supplemented with 20 % calf serum for use as a growth medium. The three suspensions : tumor cells only, tumor cells plus sensitized spleen cells, and tumor cells plus normal spleen cells, were planted in Leighton tubes on cover slips, and incubated at 37 °C. The number of cells planted per tube was 10^5 for tumor cells and 2×10^5 for spleen cells. These cover slips were removed at 24 hours in (A) or at 48 hours in (B), and stained with May-Gruenwald-Giemsa stain.

Results

It was observed that the sensitized spleen cells, especially 4 times tumor-sensitized cells aggregated around the MH 134 cells as shown in Figs. 6, 7 and 9. Most of the normal spleen cells showed no tendency to aggregate, as shown in Figs. 8 and 10.

Fig. 6 Spleen cells from BALB/C mice immunized once against MH 134 cells aggregate around MH 134 cells. (×100)

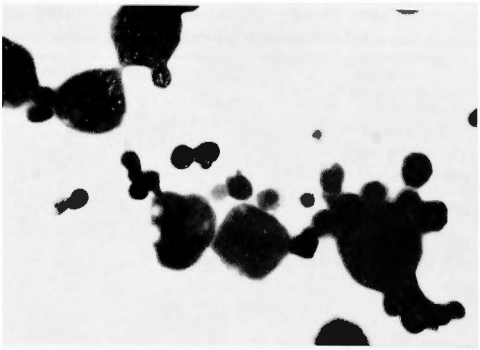

Fig. 7 Spleen cells from BALB/C mice immunized 4 times against MH 134 cells aggregate markedly around MH 134 cells. (×100)

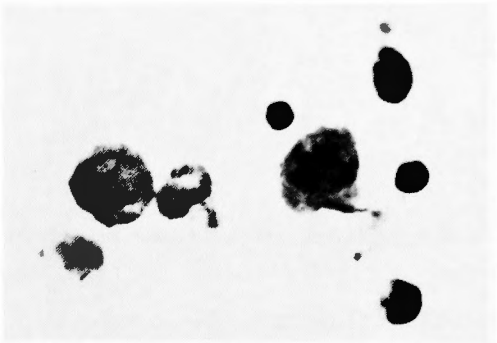

Fig. 8 Non-sensitized spleen cells from BALB/C mice show no tendency to aggregate. (×100)

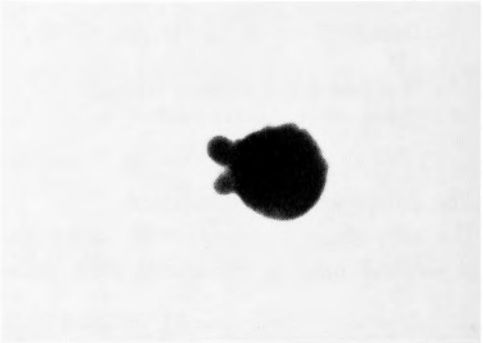

Fig. 9 BCG-sensitized spleen cells from C3H mice show slight aggregation around one MH 134 cell. (×100)

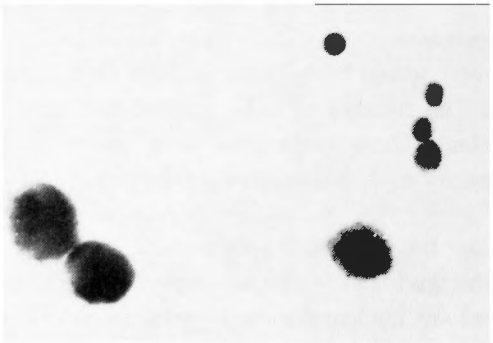

Fig. 10 Non-sensitized spleen cells from C3H mice show no tendency to aggregate. (×100)

VII. DISCUSSION

Since early in the twentieth century, all the standard immunological approaches have been tried for cancer. According to HADDOW[46], the immunological research on cancer can be divided into three phases, and the revival of cancer immunology has been developed by GORER. Furth[37] and WOODRUFF[128] also have divided immunological research in neoplasia into three eras. The first era was ushered in by the discovery of Ehrlich on immunity to grafted homologous tumor, but efforts at serotherapy, immunization with live or killed cells, and vaccines failed because of inadequate methods according to WOGLM's review as described by HATTLER and AMOS[51]. WOODRUFF has praised J. B. MURPHY's observation in 1926 on the role of the lymphocyte in resistance to tumor. The second era began with the cultivation of inbred animals and culnimated in the discovery of X-antigens and X-antibodies[41] in the serum ; KALISS[63] reported the immunological enhancement by serum antibodies. The third era began with the discovery of tumor-specific antigen in autochthonous methyl-cholanthrene-induced sarcoma by FOLEY[33] in 1953, and later by PREHM and MAIN[104], by the method of tumor graft rejection within the inbred strain of mice. Subsequently, KLEIN and SJÖGREN[55], and KLEIN et al.[66] clearly demonstrated that these autochthonous tumor cells were recognized as foreign by lymph node cells, but not by serum. Thereafter, tumor-specific antigen of virus-induced, chemically or physically induced tumors were confirmed and reviewed by OLD and BOYSE[99][100], and HATTLER and AMOS[51]. HIRAI[53] has tried chemically to analyse tumor-specific antigen in rat ascites hepatoma.

As a tool for the detection of tumor-specific antigen, JOHNSSON and SJÖGREN[62] used a tumor transplantation method in which lymphoid cells played important roles. According to KLEIN et al.[66] resistance against tumor by lymph node cells was broken down by the increase of tumor cells, and MIKULSKA et al.[84] also reported the exhaustion of resistance in spleen cells in large tumors. Hence, the treatment of depressed immunological reactivity in tumor-bearing hosts is very important.

On the other hand, antitumor drugs in large doses inhibit the immune responses such as humoral antibody production[4][21][38][79], and immune responses mediated by lymphoid cells[4][17][18][34][42][48]. Immunity depression by thymectomy[73][88][97] and carcinogen 3-methylcholanthrene[103] can promote the growth of tumors. When host defenses against the tumor can be inferred[30][80], immunity depression may have adverse effects as pointed out by KONDO[70]. In the author's experiment, mitomycin C and nitromin inhibited the production of hemolysin in C3H mice and delayed hypersensitivity response in guinea pigs, and his observations on humoral antibody support the views by FUTONAKA et al.[38], KONDO et al.[71], NOGUCHI, T.[95] and NOMOTO[96] on 7 S antibody. AMIEL et al.[4], and HITCHINGS and ELION[54] have demonstrated definite inhibition on antibody production by alkylating agents, and also in the author's experiment mitomycin C before antigen inhibited antibody production somewhat more than did nitromin. The possibility of different mechanism between nitromin and mitomycin C on immunity depression may exist.

It is generally accepted that delayed hypersensitivity in immune responses is very similar to the homograft rejection reaction[19][61][74]. As FRIEDMAN et al.[34] have reported, suppression of the delayed hypersensitivity may be a useful tool in the investigation of

the rejection of homografts. BLCOOM et al.[17] have demonstrated that mitomycin C inhibited the cellular transfer of hypersensitivity. Recently HASHIMOTO et al.[48] have clearly demonstrated the inhibitory effects of carcinostatic agents, including mitomycin C, on antitumor activity of sensitized lymphoid cells. These informations may show an importance of preventing host in cancer chemotherapy from the immunological depression which may promote tumor growth.

In tumor-bearing hosts, some researchers have reported depression of the capacity to produce serum antibodies[50)78] and many have reported depression of the immunological responses mediated by lymphoid cells such as homograft rejection reaction[26)35)40)44)57)72)77)82)83)117)120] and delayed hypersensitivity[45)52)57)76)116]. MUKULSKA et al.[84] have clearly demonstrated that the immunological reaction proved in tumor-bearing rats become exhausted by larger tumors. Erythema after the transfer of lymphocytes has been studied, and a weak reaction has been confirmed in cancer patients[5)52)108]. SOUTHAM[118] has shown that immunological defects of lymphoid cells are more important than those of serum antibodies. The author has also demonstrated an impairment of the homograft reaction in tumor-bearing mice. On the basis of these findings, it is quite possible that, assuming the very slight antigenic difference between cancer cells and normal cells, the immunological reaction would soon be exhausted or develop into tolerance in the broad sense. The thymus involution discovered by FUKUOKA and NAKAHARA[36] may be related to the defect of cellular immunity in tumor-bearing hosts. Therefore the surgical resection of tumor may have a significance in the improvement of immunological capacity, as pointed out by KOLDOVSKY[67] and APFFEL[8].

Many researchers have used infection with tubercle bacilli[30)122], e.g., BCG to reinforce the immunological capacity, and has been demonstrated to be effective against transplanted tumor by OLD et al.[101], BIOZZI et al.[16], HATA[50], NAGANO[93] and INOOKA[56]. But CRUSE and CROCKER[23] have described the allogeneic tumor graft enhancement by high titers of humoral antibody with Freund's complete adjuvant. The efficacy of tuberculosis sensitization in suppressing isogeneic tumor growth is demonstrated by WEISS et al.[127], ASHIKAWA et al.[10], and OLD et al.[98], or in tumor production by STEINKULLER and BURTON[121] and NILSON et al.[94] This effect on tumor transplantation can be seen in the early rejection of allogeneic skin grafts. The experiments by BALNER et al.[15], and VITALE and ALLEGRETTI[125] were in agreement with this concept, but RAPPORT and CHASE[106] have reported its inefficacy. As tumor specific antigens were demonstrated, the tumor suppressing effect of BCG was clearly demonstrated in chemically induced tumors by OLD et al.[98] and in virus-induced tumors by LEMONDE and CLODE-HYDE[75]. The author's experiment also shows the suppressing effect of BCG on the graph, but only three vector analysis in a time series did not show its significance. Further studies on the dose of BCG or on another activator such as described in WOODRUFF's method[129] would be necessary.

The failure of the host to react more often and more effectively against antigenic tumors, is called by HADDOW[46] as an "immunological paradox". ISHIBASHI[57] explains it by the development of immunological tolerance, and his group[58] has experimentally abolished tolerance to MM 2 cells. It seems very probable that the immunological depression accelerates development of host tolerance. From the standpoint of therapy the reduction of such tolerance is very important.

Altered antigens have been used by WEIGLE[126] and immunologically competent cells by BILLINGHAM et al.[12] and PERKINS et al.[102] to reduce this host tolerance. These methods have been used in observations of tumor growth. The resistance against the transplantation of Ehrlich carcinoma cells has been demonstrated by YOSHIMURA and KABURAKI[133] and NAGAMATSU[92], and against Yoshida sarcoma cells by ISHIDATE et al.[60] APFFEL and ARNASON[7] have shown resistance in an isogeneic host-tumor system by pretreatment of tumor cells with iodoacetate, and CZAJKOWSKI et al.[24] have reported in an autologous system growth inhibition by treatment of tumor cells with bis-diazotized benzidine. There is a very famous experiment in producing immunity by pretreatment of irradiated tumor cells by RÉVÉSZ[107]. On the contrary, MILNER et al.[86] have stressed the danger of tumor enhancement by injection of altered cells as vaccines. In the decrease of tumon volume, APFFEL et al.[8] have succeeded in producing immunity by serial tapping.

Since the clear demonstration by MITCHISON[87] and KALISS[63] that a tumor graft is rejected not by the serum, but by lymphoid cells, the utilization of immunologically competent cells has been reported in cases human cancers[9,114,130], transplanted tumors[1,22,32,49,68,123,131] in mice, and primary tumors in rats[2,25]. The origins of these cells are bone marrow, spleen, lymph nodes, thoracic duct, blood, and peritoneal fluid. Both normal and sensitized cells are used. MILLER[85] has observed that the spleen cells are more effective than bone marrow cells in the recovery of immunity following the development of tolerance. BILLINGHAM et al.[11] have also demonstrated the adoptive immunity in spleen cells. In the present study, the author used spleen cells. The data indicated that survival was not prolonged in 5-week-old mice treated with normal or tumor-sensitized spleen cells, but that it was prolonged in 8-week-old mice. These results suggest differences in host response. Assumption of homogeneity of transplanted MH 134 cells, tolerance may develop abruptly and cell growth may be speedy in 5-week-old mice. The transferred lymphoid cells, as mentioned above, had an anti-tumor effect. Did they act directly on the tumor cells?

By injection of sensitized lymphocytes labeled with radioactive uridine and by injection of unlabeled lymphocytes in splenectomized rats or those with the R. E. S. blocked with colloidal carbon, ALEXANDER et al.[2] have clearly demonstrated that their therapeutic action in vivo relies predominantly on an indirect process.

On the other hand, there are many indications of the direct action of lymphoid cells in vitro, when tumor cells maintained for many generations[1,22,65], prepared from autochthonous tumors[55,66,84,132], or from human tumors[119], are mixed with lymphoid cells, then injected in the host. Moreover, tissue culture experiments have confirmed the direct cytotoxic action of sensitized lymphoid cells on normal[109,110] or malignant cells[47,89,90,111,132]. In the author's experiment spleen cells sensitized to MH 134 cells could inhibit the growth of cancer cells and prolong the survival time.

In the author's experiment the growth of both groups of subcutaneous tumors was inhibited when transplanted in mixed suspension, however, survival was prolonged only in the group treated with tumor-sensitized spleen cells from BALB/C mice; two sorts of spleen cells were effective in the both ascitic forms. This marked effect in the ascitic form is in agreement with the observation by ALEXANDER et al.[1] On the other hand, MATIN et al.[80] have reported nullification of chemotherapeutic effect by the concomitant administration of cortisone as an inhibitor of the immune response. Therefore, it would be signi-

ficant to prevent the depression of immunological reactivity in the host, and adopt adjuvant immunotherapy to enhance the foreign body response by immunologically competent cells.

KOLDOVSKY[67] suggests the new possibility of sensitizing in vitro specifically the patient's own immune cells against his cancer and reinjecting these cells to act against the tumor. ALEXANDER et al.[2] observed previously that the transfer of heterologous immune lymphocytes was effective indirectly against primary rat tumor, and recently they have succeeded in demonstrating the antitumor effect of RNA from immune lymphocytes.[3] In a clinical study NADLER and MOORE[91] have reported that the transfer of another patient's white blood cells, particularly lymphocytes which had been sensitized to a patient's own tumor had an antitumor effect in seven cases out of twenty-six, and they also suggested the possibility of transferring lymphocytes sensitized to cancer cells treated with iodoacetate.

Immunologically competent cells have been recently accepted as capable of damaging the homologous[47)56)90], isologous[90)111], or autologous[132] malignant target cells. This cytotoxicity is marked by aggregation of lymphoid cells on the target cells with no need of complement.

GOTO and SATO[43] have reported the successful growth of MH 134 cells only after serial exchange of medium for 50 days. Then the author aimed to observe simply the action of spleen cells for MH 134 cells by aggregation, but not by computation of the survival of cells in the primary culture. Both spleen cells sensitized by BCG and sensitized by MH 134 cells showed aggregation, but the spleen cells sensitized 4 times to MH 134 cells showed the most marked aggregation. These findings indicate that the suppression of tumor growth and the prolongation of survival time are due to this effect of spleen cells on aggregation, and suggest the immunological destruction of tumor cells after contact with spleen cells, as ROSENAU and MOON[110] have reported in another system. MÖLLER[90], however, has reported the stronger cytotoxic action of lymph node cells than of spleen cells.

VIII. SUMMARY

An attempt was made to solve the immunological puzzle of why even highly antigenic tumors can grow progressively and kill their hosts by investigating the depressed immunological reactivity and also to reinforce resistance to cancer mediated by lymphocytes.

1) The antitumor drugs, nitromin and mitomycin C inhibited hemolysin formation in C3H mice, and suppressed the delayed hypersensitivity of the skin response to ovalbumin in guinea pigs.

2) The depressed immunological reactivity of the tumor was indicated by the prolonged survival time of the skin allograft from BALB/C mice.

3) The reinforcement of host resistance was investigated by sensitization to dry BCG, transfer of spleen cells, or transplantation of the mixed suspension of tumor cells and spleen cells. Suppression of tumor growth or prolongation of survival time was observed in 8-week-old mice after normal spleen cell transfer or transplantation of tumor cells mixed with tumor- or BCG- sensitized spleen cells.

4) The combined treatment with spleen cells and drugs was tried, and mice with both subcutaneous and ascitic forms treated with allogeneic spleen cells sensitized to MH 134 cells showed the best results.

5) In vitro, reactions between these spleen cells and tumor cells were observed as aggregation of spleen cells.

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(* Written in Japanese)

和文抄録

マウス腹水肝癌MH134の治療に関与する宿主低抗性の研究

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近年実験腫瘍の一部に癌特異抗原が立証されているが，何故か様な腫瘍でも宿主の制御を逸脱増殖して宿主を殺すかが問題となる．

若し僅かでも腫瘍に対する宿主抵抗性があるならば治療上それを利用，強化すべきである．本研究ではMH134に有効とされるmitomycin C及びnitromin或は癌自身による宿主免疫能の低下更にはリンパ系細胞による癌に対する宿主抵抗性の強化を試みた．実験の結果は次の如くであつた．

(1) mitomycin C及びnitrominによりC3Hマウスにおけるhemolysin産生能の低下及びovalbuminによる遅延型アレルギーの皮膚反応の発現抑制が観察された．

(2) MH134により皮下型及び腹水型の腫瘍を作り，その担癌C3HにBALB/Cの皮膚を移植した．担癌マウスではgraft rejectionの能力の低下が認められた．

(3) 担癌体における免疫能の増強として先ずBCGの感作を行なつた．腫瘍の軽い増殖抑制がgraphで推察されたが，平均vectorの差の検定でも平均生存日数の差の検定でも有意の差は認められなかつた．次に担癌体に脾細胞の輸入を試みた．生後8週間のマウスに於て延命効果が認められた．脾細胞と癌細胞をin vitroで接触後そのsuspensionを移殖して脾細胞の抗腫瘍性を調べた．MH134に対して免疫された脾細胞はgraph及び平均vectorの差で認められる，腫瘍増殖の抑制効果と延命効果を示し，BCG感作脾細胞でも延命効果が見られた．

(4) 脾細胞による処理とnitromin注射との併用では，脾細胞とMH134の比を2:1とした．MH134に対し免疫された脾細胞により皮下型のgraph上の増殖抑制及び皮下，腹水両型に延命効果が認められたが，BCG感作脾細胞ではgraph上，皮下型の軽い増殖抑制が見られ，延命効果は腹水型にのみ見られた．

(5) これらの脾細胞のMH 134に対する働きをin vitroで観察し，aggregationを認めた．以上のことより免疫リンパ球の腫瘍細胞に対するimmunological memoryの効果は宿主の癌に対するresistanceとして将来人癌の治療にも応用し得る可能性の1つの手がかりになるものと思われる．